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EFFECT OF CALCIUM CHLORIDE AND HEAT ON SOLUTIONS OF MIXTURES OF β-LACTOGLOBULIN AND CASEIN

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SUMMARY

The effect of heat and calcium chloride concentration on the precipitation in the ultracentrifuge of casein and β-lactoglobulin has been studied. With β-lactoglobulin alone (1% solution) at pH 6.5 with 2 m*M* calcium chloride per liter, the protein is totally precipitated after heating at 90° for 30 min. and ultracentrifuging (127,000 × G for 45 min.). With β-lactoglobulin (1%) and casein (2%) together under the same conditions, no precipitate is obtained until the calcium chloride concentration exceeds 4 m*M* per liter. Electrophoretic examination of the supernatant solution, when 50% of the total protein is precipitated (8 m*M* calcium chloride per liter), together with the insolubility of the precipitate in versene, suggested that the β-lactoglobulin is in the sediment. This conclusion was confirmed by the determination of the phosphorus to measure the casein distribution. Similar treatment of an unheated mixture showed that all of the β-lactoglobulin was in the supernatant solution. Also determined was the amount of calcium bound to the precipitated proteins, both alone and mixed. The total results support the assumption that the proteins in the mixture studied react independently and not as a complex.

In the course of studying the effect of heat and calcium chloride concentration on the precipitation of β-lactoglobulin *(9)* and casein *(10)*, it was observed that in a mixture of β-lactoglobulin and casein, the β-lactoglobulin was not precipitated with a concentration of calcium chloride that would precipitate it alone. Since several reports *(3, 6)* have indicated that β-lactoglobulin and α-casein form a complex when the two are heated together, the precipitation of the mixture of β-lactoglobulin and casein by calcium chloride was studied quantitatively, to see whether there was evidence for complex formation. The solubility studies were also supplemented with electrophoretic studies of the soluble and precipitated fractions. The effect of heat on a β-lactoglobulin-casein mixture containing no calcium was also studied electrophoretically.

MATERIALS AND METHODS

Proteins were purified and solutions prepared as previously described for β-lactoglobulin *(9)* and whole casein *(10)*. α-Casein was prepared by the urea purification method *(4)*.

The protein solutions were heated at 90° C. as described previously *(9)*. Ten milliliters of solution were heated for 30 min., then cooled to 30° C. in a constant-temperature water bath. This controlled cooling was necessary because of the reversal of the calcium caseinate precipitation *(10)*. The solutions were

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then centrifuged for 45 min. at 127,000 × G in the Model L Spinco ultracentrifuge.[2] The supernatant solution (soluble fraction) was removed by decantation, and calcium and protein concentrations were determined as described previously *(10)*. Unheated solutions were allowed to stand at room temperature for 2 hr., for maximum aggregation by the calcium, before centrifuging as described above. The total protein concentration in mixtures of β-lactoglobulin and casein was determined from the light absorption at 280 mμ. Fortunately, the absorption factors for these two proteins are about the same (0.93 and 0.85, respectively) and a weighted average factor of 0.88 was used. Thus, even if all of one of the proteins was precipitated, the maximum error in the protein determination would be about 4%. The validity of this factor for the mixture was confirmed by absorption measurements with known mixtures.

RESULTS

In Figure 1 is shown the precipitation in the ultracentrifuge of β-lactoglobulin, casein, and a mixture of the two after heating at 90° C. for 30 min. in the presence of 0.5 to 18.0 m*M* of calcium chloride per liter. It is evident that β-lactoglobulin alone is totally precipitated at a low concentration of calcium

[2] Mention of products does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

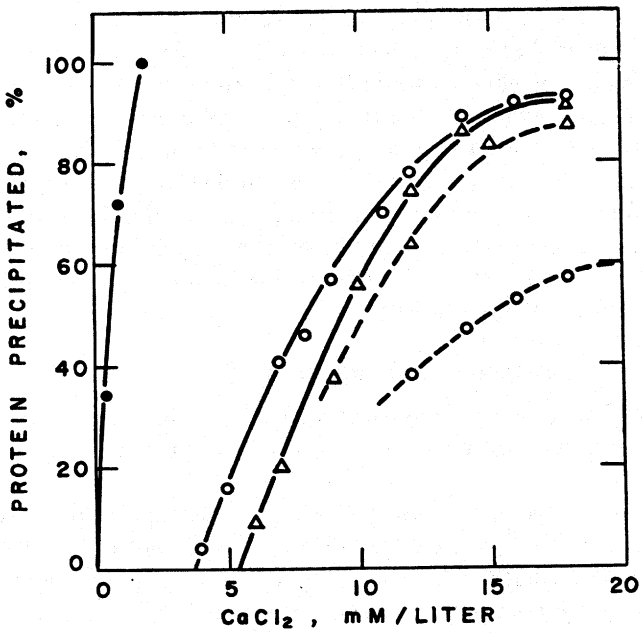

FIG. 1. Precipitation in the ultracentrifuge of β-lactoglobulin, casein, and a mixture of the two with heat (90° C. for 30 min.) and calcium chloride at pH 6.5. Also shown is the precipitation of unheated casein and a casein-β-lactoglobulin mixture with calcium chloride. Heated (solid lines): ●————●, β-Lactoglobulin (1%); Δ————Δ, Casein (2%); o————o, Casein (2%)-β-Lactoglobulin (1%) mixture. Unheated: same symbols, dashed lines.

chloride (2 m*M*/liter). In the mixture of β-lactoglobulin (1.0%) and casein (2.0%), however, a precipitate is not obtained until the concentration of calcium chloride is close to that required to precipitate casein alone.

The possibility that β-lactoglobulin might predominate in the precipitate from heated mixtures of casein and β-lactoglobulin at low concentrations of calcium chloride was suggested by the fact that in this region, the total protein precipitation with the mixture was greater than with the casein alone. To determine the composition of each fraction (precipitate and supernatant solution), a mixture of the proteins was prepared containing 8 m*M* of calcium chloride per liter and heated as before. The two fractions were separated in the ultracentrifuge. The precipitate could not be dissolved with calcium-binding versene solution. This is consistent with the idea, though not conclusive proof, that β-lactoglobulin predominates in this fraction, since calcium caseinate dissolves in the presence of versene *(10)*. The clear supernatant solution over the ultracentrifuge sediment was subjected to electrophoresis, after equilibrating with two liters of veronal buffer, pH 8.4 μ 0.1. The pattern obtained after electrophoresis for 3 hr. was that of whole casein, 27% β and 73% α, with mobilities of 3.0 and 5.8×10^{-5} cm^2/volt/sec, respectively. The concentration peaks were symmetrical and there was no evidence of β-lactoglobulin in this pattern. Thus, all of the β-lactoglobulin appears to be in the precipitate, and since about 50% of the total protein was precipitated, β-lactoglobulin composed 66% of the precipitated fraction.

The above evidence for the precipitation of β-lactoglobulin at lower calcium concentration than the casein has been substantiated by determining the distribution of phosphorus in these solutions. The phosphorus serves as a convenient tracer for locating the casein. The results of these analyses are given (Table 1). The composition of the starting mixture is 33.3% β-lactoglobulin and 66.7% casein. A solution containing 5 m*M* calcium chloride per liter, when heated and

TABLE 1

Protein composition, estimated from phosphorus content, of ultracentrifuge sediments from heated solutions containing β-lactoglobulin (1%) and casein (2%), with various concentration of calcium chloride

$CaCl_2$ conc.	Protein distribution		Phosphorus distribution[a]		Casein in sediment		
	Supernatant soln.	Sediment	Supernatant soln.	Sediment[b]	Total casein[c]	Total protein in sediment[d]	β-lactoglobulin in sediment[e]
(mM/l)	(%)						
5.0	78.6	21.4	99.9	0.1	0.1	0.3	64
6.0	60.0	40.0	96.2	3.8	3.8	6.3	113
7.0	57.1	42.9	90.0	10.0	10.0	15.6	109
8.0	48.5	51.5	80.2	19.8	19.8	25.6	115
9.0	44.8	55.2	64.2	35.8	35.8	43.3	94

[a] Total phosphorus in 10.0 ml. of starting solution was 1.54 mg.
[b] Estimated by difference.
[c] Measured by per cent phosphorus in sediment.
[d] Per cent in Column 6 times 66.7, divided by Column 3 times 100.
[e] Estimated from per cent of total protein in sediment (Column 3), less casein in sediment (Column 6 times 66.7), divided by β-lactoglobulin in 100 parts of mixture (33.3) times 100.

ultracentrifuged, has 21.4% of the total protein in the sediment. Phosphorus analyses indicate that only a negligible amount of the precipitate, namely 0.3%, is casein. With 6 m*M* calcium chloride per liter, 40% of the total protein is precipitated, and of this precipitate, 6.3% is casein and 93.7% is β-lactoglobulin. This accounts for 100% (113% by calculation) of the β-lactoglobulin. Deviations from 100% (115 to 94%) are the result of analytical errors magnified by subtracting numbers.

Although complex formation has not been suggested for unheated mixtures of β-lactoglobulin and casein, the effect of calcium chloride in precipitating such mixtures was studied to serve as a control. These results are also shown (Figure 1).

In the unheated solutions, calcium chloride precipitation of the mixture is considerably less than that of casein alone. Since unheated β-lactoglobulin itself is not precipitated with calcium chloride *(11)*, the amount of the mixture precipitated was calculated on the basis of casein alone (divide by two-thirds, since casein is two-thirds of the mixture), assuming that the binding of calcium by the β-lactoglobulin does not change the calcium concentration materially. When this is done, the amount of the casein precipitated from the mixture is approximately equivalent to that precipitated from the casein alone. An unheated mixture of 1.0% β-lactoglobulin and 2.0% casein containing 18 m*M* of calcium chloride per liter was ultracentrifuged and the two fractions were subjected to electrophoresis as described. The precipitate contained β- and α-caseins (mobilities of 3.2 and 5.9, respectively) and no β-lactoglobulin. The supernatant solution contained β-casein, β-lactoglobulin, and α-casein (mobilities of 3.2, 5.1, and 5.8, respectively).

Mixtures of β-lactoglobulin and α-casein containing calcium chloride were also studied electrophoretically for evidence of complex formation. The solution contained 0.25% β-lactoglobulin and 0.75% α-casein with 2.5 m*M* of calcium chloride per liter, and was dialyzed against two liters of cacodylic acid buffer, pH 6.5, μ 0.1, containing the same concentration of calcium chloride. When unheated, there was no evidence for complex formation, and the respective mobilities were 2.4 and 5.2 [mobilities are decreased some by the presence of calcium ions *(8)*].

When a similar solution was heated for 30 min. at 90° C. and prepared for electrophoresis in the same way, the results showed that the bulk of the protein moved with a mobility of 5.1 and that a trace moved with a mobility of about 3.1. A similar heated experiment with no calcium chloride showed the bulk of the protein moving with a mobility of 5.9 and a trace at 3.3. Thus, with or without calcium chloride these experiments appear to provide evidence for complex formation. However, the explanation appears to lie in the observation of Briggs and Hull *(1)*, that the mobility of β-lactoglobulin increases when heated under certain conditions.

A solution of 0.5% β-lactoglobulin alone at pH 7.0, heated at 90° C. for 30 min., was prepared for electrophoresis as described. Electrophoresis showed that the bulk of the protein had a mobility of 3.9, not much more than that of native

β-lactoglobulin. When heated in the presence of 0.1 *M* phosphate at the same pH, however, more than a third of the protein was transformed to a product with a mobility of 6.0. Moreover, if the heating was at 80° C., as Briggs and Hull *(1)* had observed, transformation to the higher mobility was almost complete. Thus, environmental factors make an important contribution to this transformation of β-lactoglobulin, and the presence of casein might make an added contribution.

The precipitation data shown (Figure 1) were supplemented with determinations of the calcium bound to the precipitates. The results of these determinations are shown (Figure 2) for 2% casein and for the mixture of β-lactoglobu-

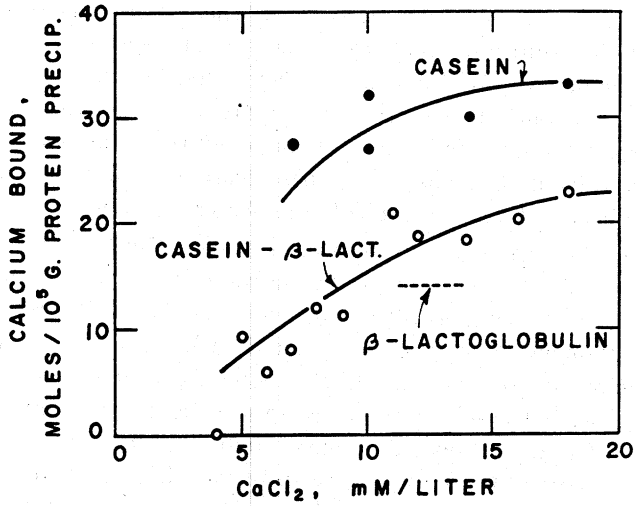

FIG. 2. Binding of calcium to casein (2%) and to a casein (2%)-β-lactoglobulin (1%) mixture at pH 6.5, precipitated with calcium chloride by heating for 30 min. at 90° C. The dashed line indicates the maximum amount of calcium bound to β-lactoglobulin at this pH.

lin and casein, heated for 30 min. at 90° C. The results favor the assumption that the β-lactoglobulin predominates in the precipitates obtained with the lowest concentrations of calcium chloride. For example, at 9 m*M* of calcium chloride per liter, where approximately 50% of both casein and the mixture are precipitated, it is apparent that considerably less calcium is bound to the precipitate of the mixture than is bound to that of the casein alone. However, if all the β-lactoglobulin is assumed to be in the precipitate, its composition would be 1 β-lactoglobulin to 0.5 casein and, using the respective binding values of 14 and 30 moles per 10^5 g., the calculated binding in the mixed precipitate is 18. This value is close to the measured binding in the mixture at this calcium concentration. Binding values for the sediments from the unheated mixtures were of the same magnitude as for casein alone. This is to be expected since, without heat, casein is the only component of the mixture that is aggregated by the calcium, and there is no evidence for complex formation between the casein and the β-lactoglobulin.

Some precipitation experiments were also done with mixtures of α-casein and β-lactoglobulin. As one might anticipate *(11)*, since the net negative charge of

α-casein is greater than the average net charge for whole casein *(5)*, about 10% more calcium is bound to α-casein than to whole casein. Contrary to expectations, however, at a given calcium concentration more α-casein than whole casein is precipitated. The calcium binding with mixtures of α-casein and β-lactoglobulin led to the same conclusions as the experiments with whole casein and β-lactoglobulin; namely, that the proteins did not interact to form a complex.

DISCUSSION

Electrophoretic experiments alone do not provide conclusive evidence as to whether β-lactoglobulin and α-casein form a complex when heated, because the heating of β-lactoglobulin solutions increases their electrophoretic mobility *(1)* until it is close to the mobility of α-casein *(6)*. Since temperature, salts, and probably other factors determine the extent to which the β-lactoglobulin is converted to a form of higher mobility when heated, the conditions for obtaining a satisfactory control are difficult to define. For these reasons, it was hoped that a study of the precipitation of the mixture of β-lactoglobulin and casein with calcium chloride could provide evidence for or against complex formation.

The precipitation studies (Figure 1) show that in the mixture β-lactoglobulin is not precipitated by a concentration of calcium chloride that will precipitate it when heated alone. Possible explanations appeared to be that the casein, by sharing the calcium chloride (the calcium-protein association constant of each is approximately the same, but casein binds more total calcium per unit weight than β-lactoglobulin), reduced the concentration available to the β-lactoglobulin below the concentration that would cause the precipitation. Or, the reduced precipitation of β-lactoglobulin might be owing to formation of a complex with the casein. One point of interest was that the mixture began to precipitate at concentrations of calcium chloride that did not precipitate casein alone. If the properties of the casein in the mixture had not changed very much, one might expect that precipitation would take more calcium, because it would now be distributed over 3 instead of 2 g. protein. This suggested that β-lactoglobulin might be the predominant component in the precipitates obtained with low calcium concentrations. The electrophoretic examination of the supernatant solution (50% of the total protein) obtained with 8 m*M* of calcium chloride per liter, together with the insolubility of the precipitate in versene, suggested that all of the β-lactoglobulin was in the sediment and composed 66% of it. This conclusion was confirmed by the determination of the phosphorus distribution. When the concentration of calcium chloride is 6 m*M* per liter, 40% of the total protein is precipitated and all the β-lactoglobulin is found in this precipitate. The total evidence indicates that β-lactoglobulin is not complexed with casein but that, instead, it precipitates independently of the casein as soon as the concentration of its share of the calcium is adequate. This interpretation of the composition of the precipitates is also supported by the relatively low amounts of bound calcium.

Experimental studies on skimmilk have not provided decisive evidence for coaggregation or complex formation. Ramsdell and Whittier *(7)* had observed

a constant difference between unheated and heated skimmilk in the amount of acid-precipitable nitrogen remaining in the supernatant as the proteins were progressively removed by centrifugation. They concluded from this that the whey proteins were not in the ultracentrifuge sediment and had, therefore, not coaggregated with the casein. Edmondson and Tarassuk *(2)* have pointed out that the nonacid-precipitable fraction will be constant, whether the heat-denatured proteins are removed in part by centrifugation or by acid precipitation alone; hence, that such experiments can not show whether coaggregation takes place. The experiments of Edmondson and Tarassuk with heated ultracentrifuge-whey suggested that the heated whey proteins are readily sedimented in the ultracentrifuge. The ability of versene to dissolve the precipitates obtained at various times of sedimentation of heated skimmilk should be a means of deciding whether whey proteins are coaggregated with the casein (versene will dissolve calcium caseinate, but not β-lactoglobulin). The phosphorus content of the sediments also might decide whether the phosphorus-free whey proteins were present.

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